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Morphological differences between Venezuelan and African microfilariae of *Onchocerca volvulus*

C. BOTTO,[1] A. ESCALANTE,[2] M. ARANGO[3] and L. YARZABAL[3]

[1]*Tropical Medicine Institute/Central University of Venezuela/UCV/Apartado 2109/Caracas;* [2]*Department of Environmental Studies/Simón Bolívar University/Sertanejas;* [3]*Amazonian Center for Research and Control of Tropical Diseases/CAICET/Puerto Ayacucho/Venezuela*

ABSTRACT

Comparative morphological and biometric characteristics of microfilariae of *Onchocerca gutturosa* and *O. volvulus* from different geographical areas (Upper Orinoco, Venezuela; Togo; Liberia) were assessed. "Stepwise" discriminant analysis and Mahalanobis estimators were applied to measure distance between populations. The results indicate a strong similarity between the two strains from the Upper Orinoco (Venezuela) and the Togo strain, as well as a clear separation between these strains and that of *O. gutturosa*. The Liberian strain was easily distinguishable from microfilariae from Togo and Venezuela. Discriminant analysis showed the Liberian deme to be as different from the Venezuelan and Togo demes as these demes were from microfilariae of the reference species, *O. gutturosa*. Although it is necessary to confirm these data using formalin-fixed specimens obtained from the skin, the present findings suggest the existence of geographically-different strains of *O. volvulus* in America and Africa.

KEY WORDS: *Onchocerca volvulus*, microfilariae, discriminant analysis, Upper Orinoco, Togo, Liberia

INTRODUCTION

Onchocerca volvulus (Nematoda, Filarioidea), the agent of human onchocerciasis or river blindness, is geographically widely-distributed, affecting 17·7 million persons on three continents (WHO, 1987). Comparative studies among populations of *O. volvulus* have demonstrated the existence of different *Simulium-Onchocerca* complexes. In Africa, apparent incompatibility has been demonstrated between strains of forest *O. volvulus* and savanna *S. damnosum* as well as between strains of savanna *O. volvulus* and forest *S. damnosum* (Duke *et al.*, 1966). Apparent incompatibility has also been noticed between African *O. volvulus* and *S. ochraceum* from Guatemala (De Leon & Duke, 1966) and between African *O. volvulus* and *S. metallicum* from Venezuela (Duke, 1970). However Guatemalan and North Venezuelan strains of *O. volvulus* have proved equally compatible with *S. metallicum* from either Guatemala or Venezuela (Takaoka *et al.*, 1986). Likewise, differences have been found in the patterns of acid phosphatase activity between African and American populations of *O. volvulus* (Omar, 1978; Yarzabal *et al.*, 1983a). Significant differences in allele frequencies by isoenzyme analysis have also been reported among *O. volvulus* from distinct geographical areas in the African continent (Cianchi *et al.*, 1985; Flockhart *et al.*, 1986). Lobos & Weiss (1985) pointed out clear antigenic differences by crossed immunoelectrophoresis between savanna and rain-forest strains of African *O. volvulus*. Erttmann *et al.* (1987) isolated a specific DNA sequence from the African forest strain of *O. volvulus* which can hybridize with several DNA isolates from the rain-forest and with at least one DNA isolate from a transition region of Togo, but fails to hybridize with DNA isolates from savanna strains.

Although comparative morphologic and chromosomal studies of both neo- and paleotropical adult *O. volvulus* failed to show consistent differences (Franz, 1980;

HIRAI *et al.*, 1987), they have been nevertheless found between African and American *O. volvulus* microfilariae (LAURENCE & SIMPSON, 1968; SCHILLER *et al.*, 1979). This combined evidence led to the hypothesis that distinct geographical strains of *O. volvulus* exist in Africa and America (DUKE *et al.*, 1966; BAIN, 1981). However, there are few studies on intraspecific morphological variation in human microfilariae (SCHACHER & GEDDAWI, 1969; COLLESS, 1971; CAMPBELL, 1976).

The current work presents data on the biometric differences between microfilariae of *O. volvulus* from America and Africa utilizing multivariant analysis, with the frame of reference being the separation between *O. volvulus* and a filaria of the same genus, *O. gutturosa*.

MATERIALS AND METHODS

The specimens of *O. volvulus* studied were from the following locations: Territorio Federal Amazonas, Venezuela, 1) Parima B, high savanna surrounded by mountainous tropical rain forest (altitude: 1050 m above sea level, 2° 44′ N, 64° 05′ W.), and 2) Orinoquito, area of low tropical rain forest on the left bank of the Orinoquito river (altitude: 250 m above sea level, 2° 25′ N., 64° 20′ W.); West Africa, 1) humid tropical forest in Liberia, and 2) savanna (transition region) of Togo.

Microfilariae of *O. gutturosa* were obtained from parasitized cattle which are pastured in the savanna of the Meta river, Apure State and in the "La Urbana" municipality of Bolivar State, Venezuela.

All microfilariae were obtained from the parasite uterus, dried on a slide and fixed in methanol in the field, and stained in the laboratory with Giemsa (Merck) diluted in a Sørensen phosphate buffer at pH 7·2 ($KH_2 PO_4$ 2·0 mM and Na_2HPO_4 12 H_2O 4·7 mM.).

Forty-one microfilariae of *O. gutturosa* and 124 microfilariae of *O. volvulus* prepared as mentioned were studied (30 from Togo, 38 from Liberia, 33 from Parima B and 23 from Orinoquito). All samples were chosen at random, and to determine the covariation of characteristics, only microfilariae were included in which all the following variables could be measured: total length, maximum width, cephalic space, position of the nerve ring, excretory cell, R1 cell, anal vesicle, last nucleus and tail space.

O. volvulus microfilariae from Liberia were frozen before fixation. As it was not possible to get fresh samples from this locality, skin snips from a patient with a heavy parasite load in Venezuela were obtained in order to study the effect of freezing on morphometric characteristics. The skin biopsies were incubated in Sørensen phosphate buffer at PH 7·2 for 12 hours. Living microfilariae were divided at random into two samples: Sample 1 was frozen immediately at −180°C in liquid nitrogen in the field. The parasites were thawed in the laboratory, fixed in methanol, and stained with Giemsa as previously described. Sample 2 was fixed with methanol in the field and stained with Giemsa in the laboratory.

In order to compare different onchocercal populations, stepwise discriminant analysis was used, with the following classification equations (BMDP Programme):

$$K=C+C_1X_1+C_2X_2+\ldots C_nX_n$$

were C is a constant, X_1 to X_n are variables and C_1 to C_n are parameters. The null hypothesis considered the parameters equal to zero (no difference among populations). Statistical significance was determined using the Fisher distribution (at a

0·05 level of significance), rejecting those variables where $F<4$. All suppositions of the model were examined (normal distribution, homogeneity of variances). The number of individuals correctly classified in each population was also estimated (Jacknife method). To know the distance among populations the Mahalanobis estimators were calculated according to:

$$D^2=(\bar{X}_1-\bar{X}_2)'\ C^{-1}(\bar{X}_1-\bar{X}_2)$$

where $\bar{X}_1$ and $\bar{X}_2$ are the vector of the means of the variables and C^{-1} is the inverse of the covariance matrix. By means of canonical analysis of populations, individuals of each population were plotted in a canonic space, where the geometric distances among points are similar to the statistical distances among individuals (CUADRAS, 1981).

RESULTS

The morphological study demonstrated the existence of a short strain of microfilaria, dominant in the sample from Liberia, which is easily differentiated from microfilariae from Venezuela and Togo. In effect, the microfilariae from Liberia are distinguished from those of Venezuela and Togo by being shorter, having a greater diameter, and a very long cephalic space ($\bar{X}=10{\cdot}27$ microns; range=5·63–19·70 microns). The nuclear column between the cephalic space and the nerve ring is very short in the Liberian strain (Fig. 1).

Of the nine variables considered, the discriminant analysis selected four: total length, maximum width, cephalic space and tail space (values of F: 104·6, 55·6, 6·6 and 4·5 respectively). The other variables, often highly correlated with total length, yielded F values less than 4·0 ($P>0{\cdot}05$) and were rejected (Table I).

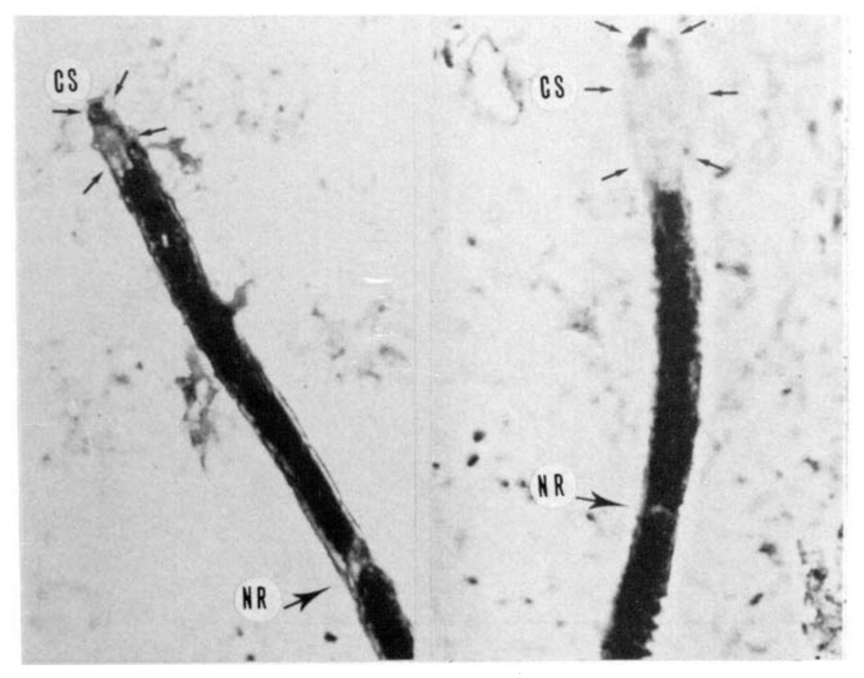


FIG. 1. *Onchocerca volvulus* microfilariae from Parima, Venezuela (left) and from Liberia (right). CS=Cephalic space; NR=Nerve ring. Phase Contrast.

TABLE I. Selected microfilarial variables (*)

		Onchocerca volvulus				*O. gutturosa*
		Venezuela		Africa		
		Parima	Orinoquito	Togo	Liberia	Venezuela
Total length	X̄	259·65	265·47	270·50	194·95	238·87
	CV	0·06	0·07	0·07	0·12	0·04
Maximum width	X̄	5·87	4·83	5·03	7·05	3·60
	CV	0·23	0·19	0·16	0·14	0·12
Cephalic space	X̄	6·90	7·02	7·61	10·27	5·02
	CV	0·13	0·15	0·19	0·36	0·34
Caudal space	X̄	12·41	11·88	10·85	9·52	8·92
	CV	0·24	0·41	0·24	0·17	0·16

(*) By Discriminant Analysis. Dimensions in microns: X̄=Mean; CV=Coefficient of variability.

The graphic projection of the canonic variables generated by these functions shows an overlapping for microfilariae of *O. volvulus* from Parima, Orinoquito and Togo, which clearly separates them from *O. gutturosa*. However, the Liberian strain is also clearly separated from those of Parima, Orinoquito and Togo as well as from the reference species, *O. gutturosa* (Fig. 2). The determination of the Mahalanobis distance confirms these findings, yielding small difference values between Venezuela and Togo and large differences between any of the other strains and that from Liberia. The last mentioned distances were of the same magnitude as those for *O. volvulus* and *O. gutturosa*. (Table II). The jacknife

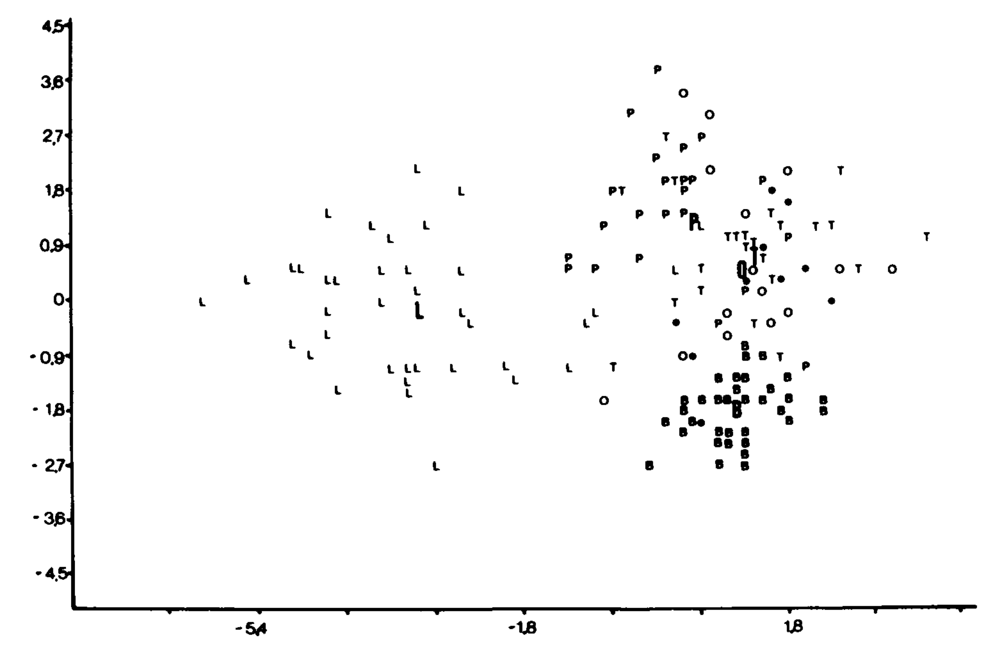


FIG. 2. Canonic variables generated by the discriminant equation: graphic projection. L=Liberia, T=Togo, P=Parima, O=Orinoquito, B=*O. gutturosa*.

TABLE II. Mahalanobis distances for microfilariae of *Onchocerca volvulus* from different localities and for those of *Onchocerca gutturosa*

	Upper Orinoco *O. volvulus*		African *O. volvulus*		
	Parima	Orinoquito	Togo	Liberia	*O. gutturosa*
Parima	0	1·6837	1·6966	3·6643	4·0922
Orinoquito	—	0	1·1039	4·5150	3·5627
Togo	—	—	0	4·1988	4·2695
Liberia	—	—	—	0	5·9634

method correctly classified 97·6% of *O. gutturosa* specimens and 86·8% of Liberian *O. volvulus* specimens. However less than 61·0% of *O. volvulus* specimens from Togo, Parima and Orinoquito were properly classified (60·6%, 50·0% and 21·7% respectively).

Frozen and unfrozen *O. volvulus* microfilariae were also compared by means of discriminant analysis, but no significant differences were found (P>0·05).

DISCUSSION

Previous studies have indicated the relevance of morphometric characteristics of microfilariae in determining species identification (Schacher & Geddawi, 1969; Bain, 1975, 1981). Moreover, it has been demonstrated that Mahalanobis distances between Guatemalan and Venezuelan populations of *O. volvulus* are at the same level when microfilariae or adult specimens are compared, and that the variance induced by different human hosts is not significant (Escalante, 1985).

A relevant finding, alluded to in a previous publication (Botto *et al.*, 1985a) is the existence of a short strain of microfilaria in Liberia, morphologically differentiable from microfilariae from Togo, Parima and Orinoquito. Discriminant analysis of the same sample clearly confirmed such difference, consistent with the Mahalanobis distance values, which indicated a difference among these strains of *O. volvulus* almost as large as the difference between *O. volvulus* and *O. gutturosa*.

Although specimens from Liberia were frozen before fixation, the results of the present study indicate that this factor does not explain the alluded differences. Gratama (1970) and Botto *et al.* (1984; 1985b) have observed that slow drying of thick smear slides produces a marked contraction of the microfilariae. However, although the total length is an important characteristic in the discriminant equation, the contraction does not account for the differences observed by us in the cephalic space or in the nuclear column before the nerve ring (Fig. 1). The low values of the coefficient of variability supports this comparison despite the small sample size. Additionally our results partially coincide with the observations made by Gibson (1952) for *O. gutturosa* and by Gratama (1970) for *O. volvulus* from Liberia.

The presence of large and small strains of *O. volvulus* microfilariae was first noticed by Blacklock (1926) in Africa and subsequently reported by others (Eichler, 1968; Gratama, 1970). It has been suggested that these may indicate a precocious sexual differentiation at the embryo stage. Martinez-Baez (1976) argues that recently emerged *O. volvulus* microfilariae may be shorter and broader than older skin microfilariae but Eichler (1968) found the opposite to be true for *O. gutturosa*. However this hypothesis does not explain our results, since the shorter strain has not been found in Togo and Venezuela. On the other hand the findings reported here strongly suggest the existence of geographically-distinct

strains of *O. volvulus* in America and Africa. The possibility that these geographical strains may differ in their pathogenicity, capacity to infect distinct vector species, and susceptibility to chemotherapeutic agents, should be carefully investigated. However, it is necessary to confirm our data using formalin-fixed, haematoxylin-stained skin microfilariae, which give more reproducible results than the Giemsa-stained microfilariae (BOTTO *et al.*, 1985b).

Discriminant analysis has been used previously for taxonomic purposes in several kinds of organisms, from protozoa (WOO & BLACK, 1984) to vertebrates (CUADRAS, 1981), but there are relatively few studies in nematodes (BLACK, 1984; ESCALANTE, 1985). We suggest that such multivariate analysis may be useful to evaluate morphological differences among filarial populations.

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